Overexpression of ECM1 Contributes to Migration and Invasion in Cholangiocarcinoma Cell

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Received October 31, 2011 / Accepted February 3, 2012

Although the expression of extracellular matrix protein-1 (ECM1) has been documented in several tumor models, the function of ECM1 has remained unclear. In this study, expression of ECM1 was detected by real time PCR and immunohistochemistry. The role and mechanism of ECM1 overexpression in cholangiocarcinoma (CCA) cells were assessed by wound-healing, matrigel invasion assay and Western blotting. Expression of ECM1 was significantly elevated in CCA tissues than that in adjacent noncancerous, cholangitis and normal bile duct tissues. Its overexpression was associated with poor differentiation, lymph node metastasis, poor prognosis, and the level of CA199, MMP-9, estrogen receptor. Knockdown of ECM1 suppressed migration and invasion of CCA cells. Using PI3K or IKK inhibitor reduced the level of phospho-Akt or phospho-IκBα as well as ECM1. Taken together, overexpression of ECM1 may contribute to CCA initiation and progression through promoting migration and invasion of CCA cells, its overexpression was associated with Akt/NF-κB signaling axis.

Key words: extracellular matrix protein 1, migration, invasion, Akt/NF-κB, cholangiocarcinoma
Follow-up was made by outpatient clinic interview, telephone or letter communication. The follow-up period was 1 year to 3 years (median 1.2 years).

Reagents. Lipofectamine2000, Lipofectamine™ RNAiMAX, Stealth-siRNA (Invitrogen); LY-294002 and BMS-345541, SB239063, Nimesulide, PD98059 (Sigma); Polyclonal antibodies against ECM1, Akt, p-Akt (Abcom), and monoclonal antibodies against MMP-2, MMP-9, ER, Her-2, β-actin, IκBα, p-IκBα (Santa Cruz Biotechnology); Goat anti-rabbit/mouse secondary antibodies, enhanced chemiluminescence (ECL) reagents (Tiangen Biotech Co); Polyvinylidene difluoride (PVDF) membranes (Millipore). Matrigel Invasion Chambers (BD Biosciences).

Cell cultures and transfection. Human CCA cell lines QBC939 and SK-Cha-1 were cultured in RPMI-1640 medium supplemented with 10% FBS, 100U/ml penicillin at 37°C in an atmosphere of air containing 5% CO2. Target stealth-siRNA (shECM1) by Lipofectamine2000.

Wound healing and matrigel invasion assays. Cells (shECM1 or shCtrl) were cultured on glass coverslips in 6-well plates. The confluent monolayers were scraped in a line across the slides with a sterile 20-μl plastic pipette tip and incubated in serum-free medium for 48h. The cell migration was evaluated by comparing the remaining cell-free area with that of the initial scrape line. Cell invasion was determined using the invaded cells were stained and counted. The invaded cells in the upper compartment of the Transwell, and 10% FBS medium was added to the lower chamber. After 48h of incubation at 37°C in a humidified CO2 incubator, non-invaded cells in the upper compartment were removed with a cotton swab, and the invaded cells were stained and counted.

Statistical analysis. All data were processed with SPSS 16.0 statistical software package. The Pearson’s χ2 were used to analyse the data from HIC. Overall survival of patients were estimated by Kaplan-Meier method. Other data were expressed as mean ± SD and analyzed by Student’s t test. Each assay was

Table 1. Relationships between ECM1 and CCA

<table>
<thead>
<tr>
<th>Features</th>
<th>N</th>
<th>ECM1 Low</th>
<th>ECM1 High</th>
<th>P</th>
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<td>Age</td>
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<td>1</td>
<td>14</td>
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<tr>
<td>W+WM</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>17</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>MP+P</td>
<td>20</td>
<td>2</td>
<td>18</td>
<td>0.736</td>
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<tr>
<td>Tumor location</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Intrahepatic</td>
<td>13</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>ExtrAhepatic</td>
<td>31</td>
<td>8</td>
<td>23</td>
<td>0.419</td>
</tr>
</tbody>
</table>

| Gallstone        |     |          |           |       |
| Yes              | 19  | 4        | 15        |       |
| No               | 25  | 8        | 17        | 0.019 |
| Lymph node metastasis | |  |           |       |
| Positive         | 27  | 4        | 23        | 0.343 |
| Negative         | 17  | 8        | 9         |       |
| Portal invasion  |     |          |           |       |
| Positive         | 17  | 6        | 11        | 0.516 |
| Negative         | 27  | 6        | 21        |       |
| Microvascular invasion | |  |           |       |
| Positive         | 15  | 5        | 10        |       |
| Negative         | 29  | 7        | 22        |       |

*P value <0.05 is considered statistically significant. W, well differentiated; WM, well-to-moderately differentiated; M, moderately differentiated; MP, moderately to poorly differentiated; P, poorly differentiated.

Consent. The clinicopathologic data of CCA patients were listed in Table 1.
repeated in triplicate in three independent experiments. Value of $P < 0.05$ was considered significant.

**Results**

**Expression of ECM1 is elevated in CCA.** To determine the expression of ECM1 in CCA, we first measured its mRNA expression by real time PCR in both CCA and their adjacent noncancerous tissues, as well as cholangitis and normal bile duct tissues. The results showed ECM1 mRNA level was significantly elevated in CCA tissues (Figure 1A). Furthermore, we detected the protein level of ECM1 by IHC which showed a predominantly, although not exclusively, cytoplasmic staining, and was not uniformly high in CCA tissues for ECM1 (Figure 1B). However, the expression of ECM1 protein was significantly elevated in CCA tissues (72.7%) compared with their adjacent noncancerous tissues (40.9%), cholangitis (41.2%) or normal bile duct (33.3%) tissues, but there was no significant difference of ECM1 expression among noncancer tissues, cholangitis and normal bile duct tissues (Table 2). Together, these results suggest that the expression of ECM1 is significantly elevated in CCA tissues.

**Overexpression of ECM1 is associated with aggressive behaviours of CCA.** Overexpression of ECM1 in CCA may contribute to the development of CCA, so we evaluated the associations between ECM1 and clinicopathological characteristics. The results from Table 1 showed that overexpression of ECM1 was significantly associated with patient sex, poor differentiation of CCA (Figure 2A), and lymph node metastasis (Figure 2B). No association was found between ECM1 and patient age, tumor location, gallstone, portal invasion, microvascular invasion. How did the ECM1 overexpression contribute to prognosis? Kaplan-Meier method was performed to assess the effect of ECM1 overexpression on overall survival. As shown in Figure 2C, overall survival rate after surgical resection was significantly worse in patients who had tumors exhibiting ECM1 high expression (median survival time, 7.9 months; cumulative 3-year survival rate, 0%) than in patients who had tumors that were low of ECM1 expression (median survival time, 32.5 months; cumulative 3-year survival rate, 21%; $P=0.005$). Furthermore, we also assessed the relationships between ECM1 overexpression and the indexes associated with CCA prognosis. The results from Table 3 showed that overexpression of ECM1 was significantly associated with CA199, MMP-9 and estrogen receptor. Taken together, these results suggest that overexpression of ECM1 may contribute to CCA initiation and progression.

**Overexpression of ECM1 contributes to migration and invasion of CCA cells.** In order to address the functional role of ECM1 in CCA cells, we tried to inhibit ECM1 expression by siRNA and tested the effect of siRNA on the proliferation of CCA cells. However, we found that downregulation of ECM1 did not affect cell proliferation (data not showed). Previous reports showed that ECM1 was associated with metastasis in several cancer cells [4,6,10]. We then used the wound-healing

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**Figure 1.** Overexpression of ECM1 in CCA tissues. (A) Total RNA samples were extracted from CCA, adjacent noncancer, cholangitis, and normal bile duct tissues. Expression of ECM1 mRNA was assessed by real-time RT-PCR. Data were normalized to GAPDH and were expressed as ratios of ECM1/GAPDH x 103. *p value less than 0.05 is considered statistically significant, which are denoted with "*". (B) Human CCAs and their adjacent noncancer tissues were immunostained with antibody against ECM1. As the arrows designate, ECM1 highly expressed in the cytoplasm of CCA cells (B1), but low or no stained in adjacent noncancer cells (B2).

**Table 2. Expression of ECM1 in different bile duct tissues**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Low</th>
<th>High</th>
<th>$P$</th>
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<tbody>
<tr>
<td>CCA(^1)</td>
<td>12</td>
<td>32</td>
<td>1 vs. 2 vs. 3 vs. 4: 0.005</td>
</tr>
<tr>
<td>Noncancer(^1)</td>
<td>26</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Cholangitis(^3)</td>
<td>10</td>
<td>7</td>
<td>2 vs. 3 vs. 4: 0.883</td>
</tr>
<tr>
<td>Normal(^4)</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Vs: Statistical analysis by chi-square test, $P <0.05$ is considered statistically significant.
assay to investigate the effect of ECM1 siRNA in migration of CCA cells. As showed in Figure 3A, downregulation of ECM1 decreased cell migration rate in SK-ChA-1, compared to control siRNA. Stable downregulation of ECM1 in shECM1 transfected CCA cells was constructed to confirm this data. Matrigel invasion assay was used to assess the migration and invasion of shECM1 and shCtrl cells. The results in Figure 3B showed that migration and invasion of SK-ChA-1 cells were significantly reduced when ECM1 was knockdowned, and a similar results were observed in QBC939 cells. These results suggest that overexpression of ECM1 plays an important role in migration and invasion of CCA cells.

Activation of Akt/NF-κB signaling axis contributes to ECM1 overexpression.

In order to determine the potential mechanism of ECM1 overexpression in CCA, we chose several signaling pathways that regulate migration and invasion of CCA cells including COX-2/PGE2, PI3K/AKT, ERK1/2, and p38 [11-13]. We examined the effects of those signaling pathways inhibition (by their inhibitors) on the expression of ECM1. Eventually, we found that suppression of PI3K/AKT by LY294002 (48h) reduced level of phospho-AKT and ECM1, but without affecting total AKT level (Figure 4A). We next studied whether activation of NF-κB, a general downstream target of PI3K/AKT, was required for overexpression of ECM1. As showed in figure 4B, IKK inhibitor BMS-345541 could reduce the level of phospho-IκBα and ECM1. Taken together, the results suggest that activation of Akt/NF-κB signaling axis may contribute to ECM1 overexpression.

Discussion

Our results showed that the level of ECM1 mRNA and protein were significantly elevated in a majority of CCA tis-
Figure 3. Effect of ECM1 knockdown on motility and invasion of the CCA cells.
(A) Downregulation of ECM1 in SK-ChA-1 cells reduced migration in wound healing assay. The confluent monolayers of CCA cells were scratched and allowed to migrate up to 48 h. The remaining cell-free area in siCtrl is less than siECM1. (B) Knockdown of ECM1 in SK-ChA-1 cells reduced migration and invasion in matrigel invasion assay. Equal cells for shCtrl and shECM1 were allowed to invade through the matrigel up to 60h or migrate through membrane without matrigel up to 48h. Invaded cells were stained with crystal violet and counted to quantify. Migration and invasion figures of QBC939 were not shown. (C) Data from (B) are expressed as percent change (means ± SEM) compared to control. Western blot analysis of ECM1 expression in shECM1 and shCtrl cells. *P<0.05.

Figure 4. Effect of Akt/NF-κB signaling axis on the ECM1 expression. The SK-ChA-1 cells were treated with or without the PI3K inhibitor LY294002 (15 μmol/L) (A) or IKK inhibitor BMS-345541 (15 μmol/L) (B) for 48h. Phosphorylated and total levels of both Akt and IkBα were analyzed by Western blotting. The results shown are representatives of three separate experiment.
overexpression of ECM1 was not associated with portal invasion of CCA cells. This is consistent with the result from the proliferation of CCA cells, suppressed the migration and invasion of CCA cells.

show that downregulation of ECM1 expression, didn’t affect the proliferation of chicken eggs [16], and its interaction with various skin tissues. We showed that overexpression of ECM1 was associated with the patient sex, poor differentiation of CCA, and lymph node metastasis, as well as several indexes of CCA prognosis [14-15]. There was a general inverse trend between a reduction in patient survival and increasing ECM1 staining intensity, which indicated that patients with high expression of ECM1 had significantly poorer survival. Therefore, it is proposed that overexpression of ECM1 may contribute to CCA initiation and progression.

ECM1 exerts its carcinogenic activity in several tumor models through promoting angiogenesis, invasion and metastasis in several cancer models [6,10]. Its metastatic-promoting properties have been suggested by its ability to stimulate the formation of blood vessels in the choroidal membrane of chicken eggs [16], and its interaction with various skin extracellular matrix proteins, such as the heparin sulphate proteoglycan perlecán, fibulin-1C/D, MMP-9, fibronectin, laminin 332 and collagen type IV [17-18]. Data presented here show that downregulation of ECM1 expression, didn’t affect the proliferation of CCA cells, suppressed the migration and invasion of CCA cells. This is consistent with the result from the clinical data that lymph-node metastases are more likely to be ECM1-positive than that without metastases, although overexpression of ECM1 was not associated with portal invasion and microvascular invasion (maybe due to small sample size). Thus, concluding from previous studies and our investigation, we suggest that overexpression of ECM1 contributed to CCA initiation and progression through promoting the migration and invasion of CCA cells.

Although previous studies have identified that ECM1 was elevated in several human tumors, the exact mechanisms that regulates ECM1 overexpression are still unclear. Previous reports showed that ECM1 was induced by overexpression of AP2alpaha in breast cancer and activation of wnt-1/beta-catenin signaling in mouse mammary epithelial cells [19-20]. However, in our study showed that suppressed PI3K/AKT pathway or NF-kB signaling pathway by their specific inhibitors reduced ECM1 expression. Previous study reported that activated PI3K/AKT pathway is associated with high proliferation index, lymph node metastasis and vascular invasion, advanced tumor stage and poor prognosis in CCA, and NF-kB is often its general downstream single pathway [12], so we propose that overexpression of ECM1 in CCA cells maybe associated with activation of Akt/NF-kB signaling axis.

In summary, our results highlights the crucial role of ECM1 in the development of human CCA by promoting the cell migration and invasion, and its overexpression maybe associated with activation of Akt/NF-kB signaling axis. Our interesting findings suggest that ECM1 may represent a therapeutic target for this devastating malignancy.

Acknowledgments: We thank all colleagues in the department of Hepatobiliary Surgery of Xiamen Hospital of Traditional Chinese Medical for collecting all kinds of bile duct tissues and the clinico-pathological and follow-up data.

Table 3. Relationships between ECM1 and factors affecting CCA prognosis

<table>
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</tr>
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</tr>
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<td>≥15</td>
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<tr>
<td>CA199(U/ml)</td>
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<tr>
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<tr>
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*P value <0.05 is considered statistically significant.

The threshold of CEA and CA199 were refered to reference 14.

References


